STUDIES ON THE RESISTANCE OF THE STRAWBERRY POWDERY MILDEW (*PODOSPHAERA MACULARIS*)

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Tiivistelmä — Referat — Abstract

Mansikanhärmä (*Podosphaera macularis*) aiheuttaa satotappioita niin kasvihuoneessa kuin avomaalla. Härmänkestävyys on eräs ominaisuus, johon mansikan jalostuksessa pyritään. Härmänkestävyyden toteaminen laboratoriokokein lajikkeiden jalostusvaiheessa nopeuttaisi härmänkestävien kantojen erottamista härmänherkistä. Tämän tutkimuksen tarkoituksena oli verrata laboratorio- ja kasvihuonekokeilla havainnollistettua mansikan härmänkestävyyttä, ja arvioida tulosten vertailukelpoisuutta, sekä metodien toimivuutta. Lisäksi selvitettiin ahomansikan F_2 polven risteytyspopulaatioista, onko härmänkestävyydellä geneettistä kytköstä rönsyjen muodostukseen tai jatkuvasatoisuuteen.

Härmänkestävyyttä arvioitiin laboratoriokokein puutarha- ja ahomansikalla inokuloimalla *in vitro*-kasvatettujen taimien lehdyköitä, ja pitämällä niitä kaksi viikkoa kostutetulla suodatinpaperilla suljetuissa Petri-maljoissa. Härmän oireet arvioitiin sitten silmämääräisesti lehdyköiltä. Puutarha- ja ahomansikan taimia altistettiin kasvihuoneessa härmän infektiolle, ja oireiden määrää lehdillä seurattiin kahdeksan viikon ajan. Puutarhamansikalla lajikkeiden välisiä eroja verrattiin laboratoriokokeissa todettuihin eroihin. Ahomansikalla havainnoitiin F_2 polven kasviyksilöiden välisiä eroja, sekä testattiin rönsyjen muodostuksen ja jatkuvasatoisuuden kytköstä todettuun härmänkestävyyteen. Rönsyjen muodostus havainnoitiin taimista kasvihuoneessa, ja jatkuvasatoisuus selvitettiin spesifisten markkereiden avulla laboratoriossa

Puutarhamansikalla laboratorio- ja kasvihuonekokeiden vertailukelpoisuus todettiin huonoksi. Laboratoriokokeiden tulokset vaihtelivat suuresti, johtuen hankalasti hallittavista olosuhteista, sekä inokulaatiomateriaalin laadun vaihteluista. Ahomansikalla laboratorioarvioinnit eivät tuottaneet ollenkaan tuloksia. Ahomansikan kasvihuonekokeissa havaittiin vaihtelua härmänkestävyydessä kasviyksilöiden välillä. Härmänkestävyyden erojen kytköstä ei pystytty tilastotieteellisesti liittämään jatkuvasatoisuuteen tai rönsynmuodostukseen, mutta mahdollisuus heikkoon kytkökseen rönsynmuodostuksen ja härmänkestävyyden välillä voitiin todeta.

Tutkittaessa härmänkestävyyttä olosuhteiden vakioinnilla, sekä inokulaatiomateriaalin laadulla on suuri merkitys laboratoriokokeiden onnistumisen kannalta. Kasvihuoneessa tautipaine pitäisi pystyä pitämään rajoitettuna, jotta erot härmänkestävyydessä lajikkeiden välillä pystyttäisiin havaitsemaan. Härmänkestävyyden ja rönsynmuodostuksen yhteyttä voitaisiin tulevaisuudessa tutkia suuremmalla määrällä kasveja.

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Tiivistelmä — Referat — Abstract
The powdery mildew of strawberry (*Podosphaera macularis*) causes yield losses in both fields and under glass. The ability to resist the infection of powdery mildew is a desirable attribute in the breeding of new cultivars. The assessment of powdery mildew resistance of cultivars by laboratory tests would give tools to quickly separate resistant cultivars from susceptible in the process of plant breeding. The purpose of this study was to compare the powdery mildew resistance of strawberry cultivars demonstrated in laboratory and greenhouse tests, and to evaluate the methods used. In addition, it was tested in the F_2 crossing populations of wild strawberry if the ability to resist powdery mildew could be genetically linked to a phenotypic character of runner production or the flowering habit.

The ability to resist powdery mildew of the garden and wild strawberry was assessed in laboratory tests by inoculating detached leaflets of *in vitro* grown plants with conidia of powdery mildew, and incubating them in closed Petri-dishes with moist filter paper for two weeks. The symptoms of powdery mildew were assessed visually from the leaflets. Garden and wild strawberry plants were exposed to powdery mildew in the greenhouse, and the symptoms were assessed during an eight-week period. The detected differences between the garden strawberry cultivars were compared to the laboratory results. The differences between individual plants were observed in the F_2 crossing populations of wild strawberry. The runnering/non-runnering trait and the flowering habit were also defined, and the connection of these traits to the powdery mildew resistance was tested. Runnering was observed from the plants in the greenhouse, and the flowering habit was determined in the laboratory with specific markers.

The comparability between the laboratory and greenhouse tests of garden strawberry was poor. The variance in the laboratory data was great due to the difficulties in the controlling of the conditions, and the quality of the inoculum. Probably because of these problems the laboratory tests on the wild strawberry failed completely. In the greenhouse there could be seen differences between the individual plants of the F_2 populations of wild strawberry. The differences were not able to be statistically linked to the habit of runner production or the flowering habit, but a hint of a weak connection could be observed between the runner production and the powdery mildew resistance.

Stable conditions and the quality and freshness of the inoculum have a great role in the success of laboratory tests in assessing powdery mildew resistance. In the greenhouse the disease pressure should be kept limited, so that the differences in the resistance to powdery mildew can be assessed. The connection between the runner production and the powdery mildew resistance could be experimented in the future with a bigger test group.

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Strawberry powdery mildew, *Podosphaera macularis*, powdery mildew resistance

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Supervisor: Timo Hytönen

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ABBREVIATIONS AND SYMBOLS

RH	Relative humidity
Fv	*Fragaria vesca*, wild strawberry
H4	*F. vesca*, accession Hawaii-4
Baron	*F. vesca*, accession Baron Solemacher

1 INTRODUCTION

The garden strawberry (*Fragaria x ananassa*) is cultivated and appreciated worldwide because of its sweet, tasty and versatile fruit. The garden strawberry can be cultivated in very different climates, and it is able to give successful yield as north as in Utsjoki, Finland (69° 54′ 25″ N). However, everywhere the strawberry is cultivated yield decreasing plant diseases are also present. Biggest field losses on strawberry are often caused by fungal pathogens. For example, the grey mold fruit rot caused by *Botrytis cinerea* is present everywhere the strawberry is cultivated, and can cause severe yield losses (Maas 1984). Like grey mold, the strawberry powdery mildew is also present in all areas of strawberry cultivation (Peries 1962). The strawberry mildew is caused by obligate parasitic pathogen *Podosphaera macularis* (Wallr.) U. Braun and S. Takam. that belongs to a group of pathogens called the powdery mildews.

The ability to resist powdery mildew varies highly between different strawberry cultivars. This variability between cultivars was first reported by Berkeley (1854, ref. Nelson et al. 1996). Many commercially popular strawberry cultivars are susceptible to the strawberry mildew, and fungicide treatments are important in the control of the disease (Hückelhoven 2005). The downside of the chemical powdery mildew control is its high price and the timing of applications; usually the biggest need for control is during harvest. Thus, using cultivars that are resistant to powdery mildew could be a better option, not only from an economical point of view, but also in order to prevent fungicide residues in the yield.

In the research of powdery mildew one important area of investigation is the improving of study methods in assessing the resistance to the disease. New strawberry cultivars are constantly being bred and the ability to resist powdery mildew is one of the important properties that determine whether the cultivar is suitable for cultivation. Even though the methods have been studied for example by Peries (1962a and 1962b), the results have been somewhat indefinite. It has, however, been shown that the resistance of strawberry to powdery mildew in field conditions can be consistent to the resistance seen in greenhouse conditions (Nelson et al. 1996). Field and greenhouse experiments are nevertheless relatively effortful to arrange, and they consume a lot of space. If experiments performed in the laboratory on Petri-dishes could give results congruent to

the results from greenhouse and field tests, it would be possible to save time and effort by quickly excluding highly susceptible cultivars from breeding programs.

One possible direction for powdery mildew studies is also the study of the genes that control the resistance and susceptibility to the disease, and the possible use of these genes in developing cultivars resistant to powdery mildew for commercial purposes. The location of the genes controlling the resistance and susceptibility can be investigated by mapping the inheritance of resistance to powdery mildew in crossing populations. The inheritance of powdery mildew resistance has been studied since the 1950's and since then a number of studies have been published (Harland and King 1957, Daubney 1961, Hsu et al. 1969, Simpson 1987).

In the summer of 2011, with the guidance of postdoctoral researcher Timo Hytönen, I did a series of laboratory and greenhouse experiments at the Department of Agricultural Sciences in Viikki, Helsinki. My plan was to screen four cultivars of the garden strawberry for their relative resistance to the strawberry mildew, and to assess and compare different research methods. In addition, I did laboratory and greenhouse tests on the F_2 crossing populations of the wild strawberry (*Fragaria vesca*), screening for the differences in the relative resistance to strawberry mildew between individual plants. In this pro gradu study I present the results and evaluate their usefulness in the studying of the ability of strawberry to resist the powdery mildew.

2 BIOLOGY OF THE POWDERY MILDEW OF STRAWBERRY

2.1 Taxonomy

The causal organism of the strawberry powdery mildew *Podosphaera macularis* (syn. *Spareotheca macularis*) is a true ascomycete fungus, belonging to the class of Leotiomycetes and order Erysiphales with one family, the Erysiphaceae. In the late 19th and during the 20th century the taxonomy of powdery mildews was based on the structure of the ascomata, but in the late 20th century technical progress allowed new

kinds of methods to be used in the investigation of taxonomical systems. Based on molecular and scanning electron microscopy (SEM) a new system of the Erysiphales was formed in the beginning of the 21st century. In the new system the genus *Spareotheca* was reduced to synonymy with *Podosphaera* (Braun et al. 2002).

In the beginning of the 20th century Salmon (1900 ref. Peries 1962) pointed out that about a hundred species of the *Rosaceae* family were hosts to a fungus morphologically identical with strawberry mildew. Transferability of traits between genera was not then reported. Peries (1962) managed to transfer conidia of strawberry mildew to *Potentilla fragariastrum* grown continuously under greenhouse conditions, but the transfer to twenty four other different species of weeds and cultivated plants failed. His results indicated that the fungus on strawberries is a specialized form of the pathogen.

2.2 Infection and symptom development

P. macularis, like other causal organisms of powdery mildews, is a biotrophic pathogen, meaning that it needs living host tissues to grow and reproduce. Because of this, the powdery mildew fungi don’t grow in axenic cultures and are difficult to study on the molecular level. This also makes storing of the fungus difficult.

The powdery mildews cause symptoms on a large group of commercially significant crops and ornamental plants, for example wheat, hops and roses. They can cause reduction of yield from 30 % up to 100% depending on the plant species (Hückelhoven 2005, Peetz 2008). Complete ruin of the strawberry yield has not been noticed, but according to Horn (1972, ref. Nelson et al. 1994) yield losses up to 60% have been reported in the United States.

2.2.1 Symptoms

The powdery mildew fungi infect the aerial parts of higher plants. The disease is most apparent as a foliar disease, but in addition to causing visible symptoms on leaves it also infects petioles, stolons, flowers and fruits. Yield losses caused by foliar damages are based on dense mycelium coverage on the leaves leading to reduced photosynthetic capacity. This can be followed by necrosis and eventual defoliation. Maas (1984) described in his book the characteristic leaf symptoms caused by the powdery mildew of strawberry. It was mentioned, that on serious infections there are white patches of mycelium developing on the lower surfaces of the leaves that enlarge and finally cover the entire lower surface. Gadoury et al. (2007b) noticed however, that the upper and lower surfaces of the leaves do not have any difference in susceptibility to the disease, and that the symptoms can just as well appear on the upper leaf surfaces. As the disease progresses the leaves may curl upwards and form reddish blotches on the surfaces (Corke and Jordan 1978).

Especially on susceptible cultivars, flowers and fruits are vulnerable to infection at all stages of development. Occasionally the strawberry powdery mildew causes serious cases of fruit rot that can also inflict yield losses. The infected flowers may then become enveloped in mycelium, which can lead to either deformation or death of the fruit. On immature fruits the mycelium causes the fruit to become hard and unable to ripen normally. Mature fruits on the other hand remain soft and pulpy (Maas 1984), have a shortened shelf life and possess small seeds (Corke and Jordan 1978).

2.2.2 Infection cycle

When the conidia of a powdery mildew fungus land on the surface of a host plant, a series of complex processes is initiated. If the host is susceptible and environmental conditions are favorable for disease development, these processes lead to a successful invasion of the host tissues, and reproduction of conidia. At first the conidia needs to attach itself to the surface, germinate, and penetrate the host cuticle and cell wall. The penetration happens by means of appressorium, a special penetration organ that is

formed by swelling from the tip of the germ tube. In optimal conditions on strawberry leaves, the conidia of *P. macularis* germinate after 4 hours from landing and appressoria are usually formed after 12 hours (Corke and Jordan 1978). The appressoria penetrate the host cell wall by means of both enzymatic and mechanical force (Green 2002). The host, however, is able to put up cell-wall associated defense, and even on a susceptible host only a portion of germinated conidia are successful in penetrating the cuticle (Hückelhoven 2005).

After successfully piercing through the cell wall, according to their biotrophic nature the powdery mildew fungi develop the haustorium, a feeding organ serving to supply the fungus with nutrients. The haustoria are formed in the host epidermal cells by invaginating the host plasma membrane, keeping the host cell still intact. As the colony develops, many generations of haustoria are formed when the hyphae continues growing on host epidermal tissues.

In addition to cell wall associated defense, the host has other strategies to prevent fungal invasion, such as suppressing the haustorial intake of nutrients. If the attacker is able to infiltrate these defenses and grow successfully, conidiophores are formed and the production of conidia begins. The single-celled conidia of *P. macularis* consist of several vacuoles that occupy more than 50% of the conidial volume (Mitchell and McKeen 1970), and develop in chains on short conidiophores that give the disease its characteristic powdery appearance (Green et al. 2002). A pore in the septum remains in a cytoplasmic connection until the conidia is mature (Mitchell and McKeen 1970). In optimal conditions on strawberry the formation of conidiophores follow after three days from completing the penetration of the host tissues, and the production of conidia begins a day later (Corke and Jordan 1978). The release of the conidia from the conidiophores supposedly happens as a result of osmotic changes causing a sudden rise in turgor of the two end conidia (Jarvis et al. 2002).

2.3 Optimal conditions and epidemiology

It has been stated that the disease incidence of the strawberry powdery mildew is positively correlated with the presence of conidia in the air (Blanco et al. 2002). There are, however, many environmental factors effecting the development of the fungus during germination, growth, formation of conidia, and finally spore dispersal. As they are crucial for the pathogen in its ability to cause disease and spread, a big portion of studies have concentrated on estimating the optimal conditions for these stages in laboratory conditions. Studies have also been done in different crop production systems. For example Xiao et al (2001) compared the epidemics of strawberry mildew in plastic tunnel and field conditions, and found out that the conditions in plastic tunnels are more favorable to the development of the disease, but that there still remains a lot that is not clear about the epidemiology of the strawberry mildew in agricultural pathosystems. In here are summarized the most important environmental factors effecting the successful infection cycle of the powdery mildew fungi.

2.3.1 Temperature

In 1962 Peries released a series of studies on *P. macularis* as the causal organism of the powdery mildew of strawberry. In the first part of his studies Peries (1962a) discussed the biology of the fungus. In Peries' studies (1962a), the minimum temperature for germination of the conidia was 2^{o}C, the germination took place most rapidly at 20^{o}C, and the germination stopped at 35^{o}C. Although some germination could be detected at the temperatures very close to 0^{o}C, infection did not take place below 5^{o}C. The growth of the fungus was slow between 5^{o}C and 13^{o}C, and it was not able to produce conidia in under 15^{o}C. It was concluded that the general-purpose optimum temperature for infection, growth and production of conidia is between 18 – 22.5^{o}C, and that close to the optimum temperature the germination reached its maximum after 25 hours from inoculation. These results were similar to those of Peetz (2008), who estimated that on hops (*Humulus lupulus)*, the optimal temperature for the conidial production of *P. macularis* is around 25^{o}C, in both constant temperature and fluctuating temperatures.

Miller et al. (2003) also studied the effects of temperature on conidial germination of *P. macularis*, and his results were similar to Peries' (1962a) studies. However, as opposed to Peries (1962), Miller (2003) managed to detect some germination at a temperature as high as 36°C. His estimate for optimum germination temperature was near 20°C, and for pathogen development 25°C. In contrast to Peries' (1962a) studies, the germination reached a maximum by 48 hours from inoculation. Subsequent penetration and colonization of the leaf tissues took place between 24 – 48 hours from germination. The conclusions of Peries (1962a) and Miller (2003) were acknowledged by Amsalem et al. (2006) who estimated in their laboratory experiments, that the optimal temperature for conidial germination and germ tube elongation is between 15 – 25°C.

It was shown by Mahaffee et al. (2003) that on hops *P. macularis* is highly sensitive to exposure to supraconductive temperatures. Only 2 hours of exposure to 30°C at the time of inoculation caused as much as 50% reduction to infection severity, and exposure to extreme temperatures (over 39°C) could cause the death of the whole colony. Periods of favorable temperatures prior to exposure reduced this effect.

2.3.2 Humidity and free water

In his laboratory experiments Peries (1962a) studied the effect of humidity on conidial germination, infection growth and production of conidiophores and conidia of *P. macularis*. Strawberry leaves dusted with conidia were incubated 24 hours at 20°C under different conditions of humidity, and the germination was then estimated. His results indicated that the relative humidity (RH) is positively correlated with the percentage conidial germination. The highest percentage conidial germination occurred in 97% RH where 87% germination was observed. In 86% RH only 48% conidial germination occurred. At 12% RH the percentage conidial germination was as low as 17%. When observing the effects of different combinations of temperature and RH, the optimal conditions for conidial germination remained at 20°C and 97% RH. The rate of conidial germination reduced significantly if either of the two factors changed. As the range of RH and temperature for optimal conditions for conidial germination appeared to be relatively narrow, it is surprising that the humidity had no effect on the post-germination infection growth or production of conidia at any given temperature.

The conclusions of Peries (1962a) were acknowledged by Amsalem et al. (2006), whose study showed that the rate of conidial germination of *P. macularis* was biggest in temperatures between 15 and 25^{o}C, with RH higher than 75% but less than 98%. However, Jhooty and McKeen (1965) found out that even though on glass surfaces the conidia need high moisture levels to germinate satisfactorily, on host surfaces the germination is possible even under very dry conditions.

Peries (1962a) also observed the effect of free water on the fungus by spraying conidial suspension onto the leaves and then drying them under a fan. It was shown that the conidia are sensitive to longer periods of immersion in water. Relatively short periods (>5 hours) of immersion inhibited the germination of most of the conidia. Examination of the samples showed that water had a lethal effect on the conidia.

2.3.3 Light conditions

Peries (1962a) did not manage to detect any differences in the rates of conidial germination or development of the fungus in different light conditions. His results are, however, in controversy with the study of Amsalem et al. (2006) who showed that high light intensity could reduce conidial germination and hyphal growth. Incubation of conidia on leaves in complete darkness resulted in significantly higher rate of germination than under alternating dark/light regime. Differences were also observed in germ tube length that was higher on conidia germinated in the dark compared to the alternating regime.

The sensitivity of powdery mildew fungi to light was also showed by Suthaparan et al. (2009), who found out that continuous light reduces the conidial production and germination on the rose powdery mildew (*Podosphaera pannosa*). In growth chambers exposure to 24 hours of daily light could reduce the production of conidia up to 62%, in contrast to the 22% reduction in 18 hours of lighting.

Jordan and Hunter (1972) observed the differences in the performance of strawberry plants under glass cloches and clear polyethylene tunnels compared to colored polyethylene tunnels, and noticed that the strawberry powdery mildew was more severe

on plants under the colored films than under glass or clear film. It was, however, pointed out that other factors in the microclimate, such as humidity differed significantly under the various covers, and thus the results could have been due to other factors than light conditions.

2.3.4 Conidial dispersion

After the conidia are released, there are several factors effecting the dispersal and spread of the disease in the production system. A glasshouse and a plastic tunnel, for example, have different wind conditions, wind speed being about ten times lower in the glasshouse than in the tunnel. This resulted in more disease on strawberry growing in plastic tunnel than in the glasshouse in Willocquet's (et al. 2008) study, where they compared the dispersal of conidia and disease gradients in the two production systems.

Blanco et al. (2004) monitored the atmospheric concentrations of *P. macularis* conidia on field conditions in Spain. Their main results were that the disease incidence was positively correlated with the presence of conidia in the air and that the presence of conidia 40 cm above the beds was positively correlated with temperature and negatively correlated with relative humidity and rainfall. Also Peries (1962a) noticed that rain has pivotal effect on spore dispersal as it damages the conidiophores, and that their recovery may take up to three days. Sutton and Jones (1979) analyzed factors affecting dispersal of *Podosphaera leucotricha*, the causal organism for the powdery mildew of apple, and found out that in addition to temperature, the concentration of conidia in the air was positively correlated to wind velocity and solar radiation. The negative effect of humidity and leaf wetness on production of conidia was also observed.

Even though the powdery mildews are windborne diseases, the conidia are not likely to travel long distances via wind. It was noted by Peries (1962a) that most conidia of *P. macularis* are dispersed within the canopy, rather than above it. The question remains how the powdery mildews travel longer distances, for example between continents. Jarvis (2002) speculated that the conidia may be transported in the clothes of visitors on the plantations, but this is not verified by any scientific study.

2.3.5 Perennation

The ability of the powdery mildews to survive between cropping seasons is strongly dependent on the climatic factors. In greenhouse productions systems, or in areas of more temperate climates where there is basically no discontinuity in cropping, the powdery mildews can maintain active pathogenesis and dispersal, and thus are able to sustain perennial activity (Jarvis et al. 2002). The powdery mildews do not have sclerotia or other kinds of specialized survival structures. In temperate climates, in perennial fruit and vine crops the pathogen survives between cropping seasons as mycelium in the dormant buds of the crop (Jarvis et al. 2002).

The cleistotechium, the teleomorph that serves as the site of sexual reproduction can function as a perennating structure of the powdery mildew fungus to survive over periods when green host tissues are not available. In Israel's hot and rainless summer the cleistotechia of *Erysiphae graminis* (syn. *Blumeria graminis*) were discovered to be the principal, if not the only over-summering organ on barley (Koltin and Kenneth 1970), while in cold and dry winters in New York vineyards the pathogen survived the winter as cleistotechia on the bark of the crop (Gadyoury and Pearson 1988). However, the role of cleistotechia as the overwintering organ of the powdery mildews is controversial, since many times the fungus is unable to produce viable ascospores in the spring (Jarvis et al. 2002).

The role of cleistotechia in the epidemiology of strawberry powdery mildew has been unclear to researchers. Even though reports of cleistotechia found on strawberry have been made every now and then during the past decade (for example Gourley 1979 in Nova Scotia, Howard and Albregts 1982 in Florida), the assumption has been that they have no remarkable role in the life cycle of the fungus. Peries (1962a) found no evidence of the fungus being heteroallic in his tests, and Maas (1984) states in his book that the strawberry powdery mildew does not appear to over-winter as cleistotechia, but survives as mycelia in older, still living leaves.

These conclusions might be acceptable when concerning production in areas where milder winters allow some parts of the crop to stay alive until spring. However, for example, in the northern Europe winters are cold and aerial parts of the plants do not

always survive the winters, and yet disease is present again in the spring. It has been demonstrated that in perennial field production systems in Norway cleistotechia of *P. macularis* are indeed functional survival structures, and that the pathogen is heteroallic (Gadoury et al. 2007a). Furthermore, in their comprehensive study on initiation, development and survival of cleistotechia of *P. macularis,* Gadoury et al. (2009) found out that in Norway cleistotechia appear to be a functional source of primary inoculums for strawberry powdery mildew. They also found out that the cleistotechia of *P. macularis* differ from those of other powdery mildews by their propensity to remain attached to the persistent leaves of their host during the intercrop period. It is likely that the role of cleistotechia in the survival of powdery mildews is highly dependent on the climate and production system of the crop, and the nature of the crop itself.

2.4 The powdery mildew resistance of strawberry

Because the powdery mildew of strawberry is abundant where ever strawberry is cultivated, in the development of strawberry cultivars the resistance to powdery mildew is a desirable feature. High yields may even be dependent on the ability of the crop to resist powdery mildew. Thus, it is a feature that has been studied along the disease itself.

First screenings of the relative resistance of some strawberry varieties were done in the late 1950's by Orchard and van Adrechem (1957, ref. Daubeny 1961), Miller and Waldo (1957, ref. Daubeny 1961), and Daubeny (1959, ref. Daubeny 1961). In the beginning of the 1960's Peries (1962b) tested thirty-six varieties of cultivated strawberry for their foliar reaction to inoculation on powdery mildew, in both laboratory and green house conditions. It was shown, that the basis of the resistance of strawberry leaves to the infection of powdery mildew is related to the ability of the fungus to penetrate the cuticle and epidermal wall of the plant. In his laboratory tests and microscopy studies all successful cuticle penetrations by the fungus lead to further development of the infection, resulting in normal production of haustoria, mycelium and conidiophores. In Peries' studies (1962b) the necrosis of the leaves did not correlate

with the resistance to the disease. It was also observed that the younger leaves seemed to be more susceptible to the disease than older leaves.

2.4.1 Inheritance of powdery mildew resistance

Along the variety screenings, the investigation of inheritance of powdery mildew resistance has been the main target of the studies on strawberry in order to find material for future breeding programs. Harland and King (1957) studied the powdery mildew resistance in diploid *F. vesca*, and concluded that the resistance was dependent on two recessive genes, but was complicated by an unknown cytoplasmic factor. Daubeny (1961) studied the effect of various parents on the degree of powdery mildew resistance in progenies of cultivated strawberry, and suggested that the powdery mildew resistance in strawberry is controlled by relatively few genes. However, in contrast to Harland and King's (1957) studies, no maternal effect on resistance could be detected.

In 1969 (Hsu et al.) analyzed segregation of the resistance to powdery mildew in progenies of cultivated strawberry, simultaneously using the quantitative method based on continuous variation and the Mendelian method based on discontinuous variation. The quantitative analysis suggested that in the total genetic variance epistatic variance has an important role, and that non-additive variance is more important than additive variance. They also disagreed with Harland and King's (1957) suggestion that the resistance was depending on two genes only. Furthermore, even though Daubeny (1961) was unable to confirm a maternal effect on powdery mildew resistance in the progenies he studied, the Mendelian analysis of Hsu et al. (1969) in their study showed a parental contribution to resistance in 88% of the progenies. The Mendelian analyses also suggested that the segregation was dependent on two additive genes for resistance and one epistatic gene for susceptibility.

More evidence for maternal effect was found by MacLachlan (1978), whose results from studies on seedlings of cultivated strawberry indicated the existence of a maternal effect on mildew susceptibility. Simpson (1987) investigated the inheritance of the powdery mildew resistance in the strawberry progenies from crosses between short-day and either ever-bearing or day-neutral genotypes, and got results that were in

accordance with those of McLachlan (1978). Simpson also suggested that both additive and non-additive variance have an important role in the inheritance of powdery mildew resistance.

2.4.2 The effect of different environments on the powdery mildew resistance

The differences between agricultural systems may have effect on how the crop is able to respond to disease pressure. For example Xiao et al (2001) compared the epidemics of strawberry mildew in plastic tunnel and field conditions and found out that the conditions in plastic tunnels are more favorable to the development of the disease.

Peries (1962b) compared the powdery mildew resistance of strawberry between laboratory tests and greenhouse tests, and even though differences in the susceptibility to the strawberry mildew could be detected between the tested varieties, the laboratory results did not correlate with the results of the greenhouse tests. It was shown, that varieties that showed resistance to the disease in laboratory conditions could lose their resistance in the greenhouse.

Nelson, et al. (1995) studied the inheritance of powdery mildew resistance strawberry progenies grown in greenhouse and field conditions, in order to investigate the possible differences in the manifestation of the resistance in different environments. In their study genetic differences in resistance were expressed alike in both environments, suggesting that the powdery mildew resistance of cultivars in field conditions could be screened in greenhouse conditions. They also suggested that different genes may confer resistance with different levels of disease pressure. Nelson et al. (1996) continued their work by screening 47 strawberry cultivars for their relative resistance to powdery mildew in greenhouse, fruit production fields and propagation nurseries. The powdery mildew resistance was expressed similarly, but not in a completely homologous way, in all three environments, backing up the suggestion in their previous study (Nelson et al. 1995) that the expression of mildew resistance could be correlating between different cropping systems.

3 THE AIMS OF THE STUDY

The purpose of this pro gradu study was to screen four cultivars of garden strawberry (*Fragaria x ananassa*) for their relative resistance to powdery mildew (*Podosphaera macularis*) in the laboratory and in the greenhouse, and discuss the comparability of these results. The ability of the strawberry cultivars used in this study to resist the infection of the powdery mildew was known beforehand. It was aimed to find out if the relative resistance of cultivars of the garden strawberry to the strawberry powdery mildew can be screened reliably by laboratory tests, and if the results are applicable to agricultural systems such as the greenhouse. In addition, the ability to resist the powdery mildew was assessed in laboratory and greenhouse tests from individual plants of three F_2 crossing populations of the wild strawberry (*Fragaria vesca*). It was tested if the possible differences in the relative resistance between individual plants could be genetically linked to phenotypic characters of runner production or flowering habit.

It was hypothesized, that in both laboratory and greenhouse tests the cultivars of the garden strawberry known to be more resistant to the powdery mildew would prove their resistance by showing less symptoms of the disease, and that the cultivars known to be susceptible would show more symptoms. This way the laboratory tests would give reference to the hands-on powdery mildew resistance of the cultivars in the greenhouse, and could be used as quick tests to determine the field performance of the cultivars.

It was also hypothesized, that genetically controlled differences can be seen in the abilities of individual plants to resist the infection of powdery mildew in the F_2 crossing populations of the wild strawberry. That is why it was analyzed, if the susceptibility or the resistance occurring in the populations could be genetically linked to either one of the two known single-gene regulated phenotypic characters (Brown and Wareing 1965). The hypothesis is based on the expected 1:3 segregation of these phenotypes in the whole population. If this ratio differs from 1:3 in the populations of the selected resistant and susceptible individuals, the resistance/susceptibility is genetically linked to the phenotypic character in question.

4 MATERIALS AND METHODS

4.1 Experiments on garden strawberry

The differences in the resistance to strawberry powdery mildew caused by *Podosphaera macularis* between cultivars of the garden strawberry (*Fragaria x ananassa*) were studied on four cultivars, Jonsok, Valotar, Suvetar and Zefyr. The plant material for the tests was obtained as *in vitro* plants, the cultivar Zefyr from the Piikkiö experimental area of MTT Agrifood Research Finland, and cultivars Jonsok, Suvetar and Valotar from MTT's Research and Elite Plant Station in Laukaa.

The incubations for the laboratory tests were done in growth chambers in temperature of 23^{o}C and lighting period of 12/12 hours. The relative humidity of the growth chamber was not relevant to the results, because the samples were placed on Petri-dishes with closed lids. The greenhouse experiments took place in the greenhouses of the Faculty of Agriculture and Forestry in Viikki, Helsinki. The plants were grown in short day light conditions in 18^{o}C, illuminated for 12 hours daily by HPS lamps with the irradiance 120 µmol/m^{2}/s.

4.1.1 Preparation of the plant material

For the greenhouse and laboratory tests, *in vitro* plants of the garden strawberry cultivars Jonsok, Valotar, Suvetar and Zefyr, propagated from meristem cultures, were transplanted from delivery containers into round lidded glass jars, with 60 ml agar rooting medium (Appendix 1). The transplantation was done in a laminar closet by removing separate plants from the delivery container, cutting leaves and roots leaving only the smallest un-emerged leaves, and carefully placing the transplant into the rooting medium in the jars, five plants per jar. The jars were then placed inside a growth closet meant for *in vitro* plants, and let root and grow for at least six weeks.

4.1.2 Greenhouse tests

For the greenhouse tests on the garden strawberry, 30 of *in vitro* grown plants of each cultivar were moved to the greenhouse. The plants were removed from the glass jars, the agar rooting medium was washed away from the plants under running tap water, and the plants were then planted into small (5 x 5 cm) plastic pots. The soil used in the pots was a peat mixture by Kekkilä (Kekkilän kasvuseos, Kekkilä Oy, Vantaa, Finland). The peat mixture was watered thoroughly before placing it into the pots. The plants were then placed into lidded mini greenhouses, and watered every other day to keep the peat moist continuously.

After a week in the mini greenhouses the plants were moved to open tables in the greenhouse. The plants were kept in conditions where disease pressure of strawberry mildew was available and abundant from the strawberry plants already growing in the room. To make sure that the disease reached the seedlings uniformly a heavily diseased plant was shaken above the test plants and then placed to a close distance from the plants. The places of the plants were also randomized during each watering.

The plants in the greenhouse were observed after four, six and eight weeks on the open tables. All of the leaves were screened visually for relative resistance to the powdery mildew by assessing the approximate percent surface area of the leaves that was covered with visible fungal growth, such as conidia and conidiophores (sporulation). No individual plants were itemized, because single leaves were treated as observational units. After six weeks, when some of the leaves had started to decompose, the data was only collected from leaves with more than 50% living tissue. The measurements of the single leaves were then compared between the cultivars by performing an ANOVA single-factor data analysis in Microsoft Office Excel (version 2007 SP3, Microsoft Inc., Redmond, WA, USA).

4.1.3 Laboratory tests

For the laboratory tests on the garden strawberry, leaflets obtained from *in vitro* grown plants of each four cultivars were placed on Petri-dishes and inoculated with spores of *P. macularis*. Moist filter paper was put on the bottom of the Petri-dishes and seven leaflets were placed on each dish with the abaxial surface upwards, altogether three dishes and 21 leaflets per cultivar. Conidia were then gently brushed from diseased leaves on top of the leaflets, (the first round of tests with a plastic paintbrush and the rest of the rounds with a pig bristle brush), making sure that approximately same amount of inoculum landed on each leaflet. The dishes were then closed with a lid and left to incubate for two weeks. To make sure the humidity would not get too high no Parafilm was used on the dishes.

The Petri-dishes were watered during the two weeks of incubation to keep the filter paper moist. After the incubation, the symptoms on the leaflets were observed visually, by assessing the approximate surface area that showed visual changes caused by the fungus, such as red blotches or visible sporulation. Similarly to the greenhouse experiments, no individual plants were itemized, because single leaves were treated as observational units. The tests were repeated six times, on new leaflets every time. On the last round one Petri-dish of un-inoculated control samples of each cultivar were incubated along the inoculated samples.

On the first round of the laboratory tests two moist filter papers were placed on the dishes to make sure the samples stayed moist enough and thus the sample were only watered once during the two week incubation. On the rounds two to six only one filter paper per dish was used and the samples were watered every two or three days.

The measurements were then compared between the cultivars by performing an ANOVA single-factor data analysis in Microsoft Office Excel.

4.2 Experiments on wild strawberry

Variation in the powdery mildew resistance was analyzed in three *F. vesca* F_2 crossing populations: Finnish wild type (Fv) x Hawaii 4 (H4), Fv x Baron Solemacher (Baron) and Baron x H4. The seeds of the F_2 crossing populations used in the experiments were obtained from Dr. Timo Hytönen. The inoculum of the powdery mildew originated from the strawberry plants grown in the greenhouses of the Faculty of Agriculture and Forestry in Viikki.

4.2.1 Laboratory tests

The laboratory tests were performed on plants of Fv and H4, and on the Fv x H4 F_2 crossing population. The plants were propagated from seeds *in vitro*.

Approximately 200 crossing population seeds, and 100 of both Fv and H4 seeds were surface sterilized and then germinated on Petri-dishes on germination medium (Appendix 2). After forty days when the seeds had germinated and grown a pair of leaves in addition to the cotyledons, 50 seedlings of the crossing population and 10 seedlings of both Fv and H4 were moved to grow on rooting medium (Appendix 1) in lidded jars.

The laboratory tests were performed similarly to the laboratory tests on the garden strawberry. Leaflets obtained from *in vitro* grown plants of the crossing populations were placed on Petri-dishes with moist filter paper and inoculated with spores of *Podosphaera macularis*. Since the meaning of the test was to compare the ability to resist the powdery mildew infection of individual plants, each Petri-dish only had leaflets from one plant. 50 plants of the crossing population were inoculated. Four Petri-dishes of both parental genotypes (Fv and H4) were also inoculated as controls. The dishes were then closed with a lid and left to incubate.

4.2.2 Greenhouse tests

To test if differences could be seen in the ability of the plant individuals in Fv x Baron, H4 x Baron and Fv x H4 F_2 crossing populations to resist powdery mildew, plants were grown in the greenhouse. Plant material for the greenhouse tests performed on the wild strawberry was propagated in the greenhouse from seeds. In addition, parental accessions Fv, Baron and H4 were also grown.

Approximately 200 seeds of each three crossing populations and 20 seeds of each parent were sown to moist bed of Kekkilän Kylvöseos, covered with plastic and left to germinate. The germinating was done in a room with long day conditions to hasten the process. After two weeks the plastic was removed and the beds were relocated to short day conditions to prevent the flower induction. When one leaf after the cotyledons had emerged, the seedlings were moved and planted individually into small (7 x 7 x 6.2 cm) plastic pots with Kekkilän Kylvöseos. The plants were kept moist by manually watering every couple of days. The places of the seedlings were also randomized during each watering.

Because the powdery mildew was abundant in other strawberry plants in the room, no separate inoculation was done on the seedlings. After relocating into the short day room, the seedlings were observed every couple of days to detect the first visible symptoms of powdery mildew. After the first symptoms started to show, the first assessment of the amount of symptoms on the leaves was done.

In contrast to the experiments done on the garden strawberry, this time the plants were itemized, and symptoms were assessed on each plant individually. It was also taken into account on which leaf the symptoms were detected on, in such a way that the leaves were numbered in the order they had emerged. The first leaf to emerge after the cotyledons was number 1, and so on. This way the signification of the age of the leaf to the susceptibility to powdery mildew could be evaluated. The measurement procedure was repeated after two weeks.

The data obtained was then analyzed using Microsoft Office Excel. The overall stage of contamination of the individual plants was calculated by summing the percentage

coverage areas of visible symptoms of the individual leaves of the plant. Histograms were then drawn to see how the plant individuals with different percentage coverage of symptoms were distributed in the population.

4.2.3 Phenotyping the individual plants

To see if the individual plant's ability to resist powdery mildew could be liked to a phenotypic character, fifteen of the most and fifteen of the least contaminated plants of each population were taken under further investigation. The characters evaluated in these groups were the production of runners and the flowering habit. The differences in both characters are controlled by different single genes. The ability to produce runners is dominant to not producing runners, and the seasonal flowering is dominant to perpetual (everbearing) flowering. The genotypes and phenotypes of the parents considering these characters are presented in table 1.

Table 1. The genotypes and phenotypes of the parents, considering the production of runners (Character A) and the flowering habit (Character B).

	Baron	**Fv**	**H4**
Genotype	aabb	AABB	AAbb
Phenotype	Perpetual No runners	Seasonal Runners	Perpetual Runners

To experiment the possibility of a genetic link between the ability to resist powdery mildew and the phenotypic characters, it was tested if the ratio of the expressed phenotypes among the resistant and susceptible individuals differed statistically from 3:1 ratio.

In the Fv x Baron -population both the runner production and flowering habits are characterized. Both H4 and Baron are recessive homozygotes considering the flowering habit and thus all the plant individuals of the F_2 crossing population have perpetual

flowering. This is why in the H4 x Baron -population only the production of runners is characterized. In the Fv x H4 -population only the flowering habit is characterized, because both of the parents Fv and H4 are dominant homozygotes considering the production of runners.

The ability of the plants to produce runners was observed at the time of the second symptom assessment. In order to save time, the flowering habit of the plant individuals in the Fv x H4 and Fv x Baron -populations was defined in the laboratory. This was done by carrying out a fragment analysis with specific markers.

4.2.4 Fragment analysis

The DNA was isolated from the plants using the Doyle and Doyle method (1990, Appendix 3). The samples were collected from fifteen of the least and fifteen of the most contaminated plant individuals of both the Fv x H4 and Fv x Baron –crossing populations (from all together 60 plants, of which 30 were characterized as susceptible and 30 as resistant). The samples were taken by collecting a small piece from the leaf that is just about to emerge from the growth point. The samples were put into 2 ml tubes with two ball bearings and placed on dry ice. The samples were then chilled with liquid nitrogen and ground using the Oscillating Mill MM400 (Retsch Inc., Haas, Germany).

Because of the ball bearings in the tubes a few changes were made to the DNA isolation protocol. In the third step 0.8 ml of CTAB-buffer was added in the tubes instead of 0.5 ml, the tubes were centrifuged at maximum speed for 3 minutes and 0.65 ml of the supernatant was pipetted into 1.5 ml tubes. This way the ball bearings could be separated easily from the samples, and washed to be used in the future.

In the fourth step of the protocol 0.5 ml of the aqueous phase was moved into a clean tube after the first extraction. A clear phase was achieved after two extractions, and 0.4 ml of the aqueous phase was then moved into a clean tube. In the seventh step the tubes were centrifuged at maximum speed for five minutes to make sure the pellet stayed in the bottom, before removing the ethanol. The steps 8 and 9 were combined in a way that 4 µl RNaseA was mixed into 1 ml TE, and the pellets were then re-suspended in 50 µl

of the TE + RNaseA mixture. The purity and amount of DNA in the samples were then measured with GeneQuant 1300 spectrophotometer (GE Healthcare Inc., Buckinghamshire, UK) from five random samples of the most and least contaminated plants from both populations in a way that all together 20 samples were measured. For the spectrophotometer measurements a 1:100 dilutions of the samples were prepared.

A PCR was then prepared, using markers linked to the flowering habit (Table 2). 1:100 dilutions of the sampled were pipetted onto a PCR plate, and a master mix for the reaction (Appendix 4) was prepared. The PCR reaction was done with Mastercycler Gradient (Eppendorf Inc., Hamburg, Germany). The master mix was then pipetted onto a new PCR plate, and 2.5 µl of the diluted sample was added.

Table 2. The markers used in the PCR reaction.

Marker		Size of the fragment	Stamp
TFL1_lab	F + R	283 – 285 bp	HEX
PRR7	F + R	390 – 392 bp	6-FAM
CFVCT010	F + R	119 – 131 bp	HEX

After the program the success of the PCR was made sure by running a gel electrophoresis on eight random samples from the plate. The gel was prepared with 0.5 x TBE, 1.2% agar and 2.5 µg/ml ethidium bromide. The sample for the gel was prepared with 2.5 µl PCR sample, 7.5 µl MQ water and 2.5 µl loading buffer, and pipetted into the wells. In addition, 5 µl of Gene Ruler DNA Ladder (Fermentas International Inc., Burlington, Canada) was pipetted into two wells. The gel was then run for 45 minutes.

Because the genotype of ever-bearing traits differs from the genotype of annual traits only by a deletion of two base-pairs in the gene, the differences in the size of the gene fragment could not be detected by regular electrophoresis. Because of this the samples were sent to the DNA sequencing laboratory of the Institute of Biotechnology in Viikki to be analyzed by capillary-electrophoresis. In the capillary-electrophoresis the fluorescence stamp in the marker is detected. To identify the genotype of the sample

plants the results of the capillary-electrophoresis were then analyzed with PeakScanner Software (version 1.0, Life Technologies Inc., Carlsband, CA, USA).

4.2.5 The chi-square test

To find out if the ratios of the expressed phenotypes among the susceptible and resistant individuals of the Fv x Baron and Fv x H4 -populations differed significantly from 3:1, chi-square tests were done. For the chi-square tests the data of the susceptible individuals of both populations was combined into one test group, and the data of the resistant individuals into another. Thus both the segregations of the flowering habit and the runner production were analyzed separately in a group of 30 susceptible, and in a group of 30 resistant individuals.

5 RESULTS

5.1 Experiments on garden strawberry

5.1.1 Laboratory tests

The difference in the relative resistance to powdery mildew between cultivars was tested in the laboratory conditions on Petri-dishes by inoculating strawberry leaflets with conidia of strawberry mildew and then incubating the samples for two weeks. On the first round of the laboratory tests visible conidiophores could only be detected on one of the 84 leaflets inoculated. The sample leaflets started turning red after a couple of days post inoculation, but no other changes could be seen on the samples, except for one Jonsok leaflet. This is why the results from the first test round were discarded, and only the results from rounds two to six were taken into consideration.

The results between the test rounds varied greatly (Figure 1). Even though on every test round there was a statistical significance in the differences of the average amounts of symptoms between cultivars ($p<0.05$), the results were not consistent between the test rounds. The results varied greatly also within the test rounds and even within the test groups (cultivars). The range between coverage areas of symptoms on leaflets varied between 0 to 100% even inside the same dish. It was difficult to keep the conditions stable inside the dishes, and as a result on the third and fourth round there were dishes that had to be discarded because the leaflets had dried out and died. All together, one Petri-dish of each cultivar had to be discarded.

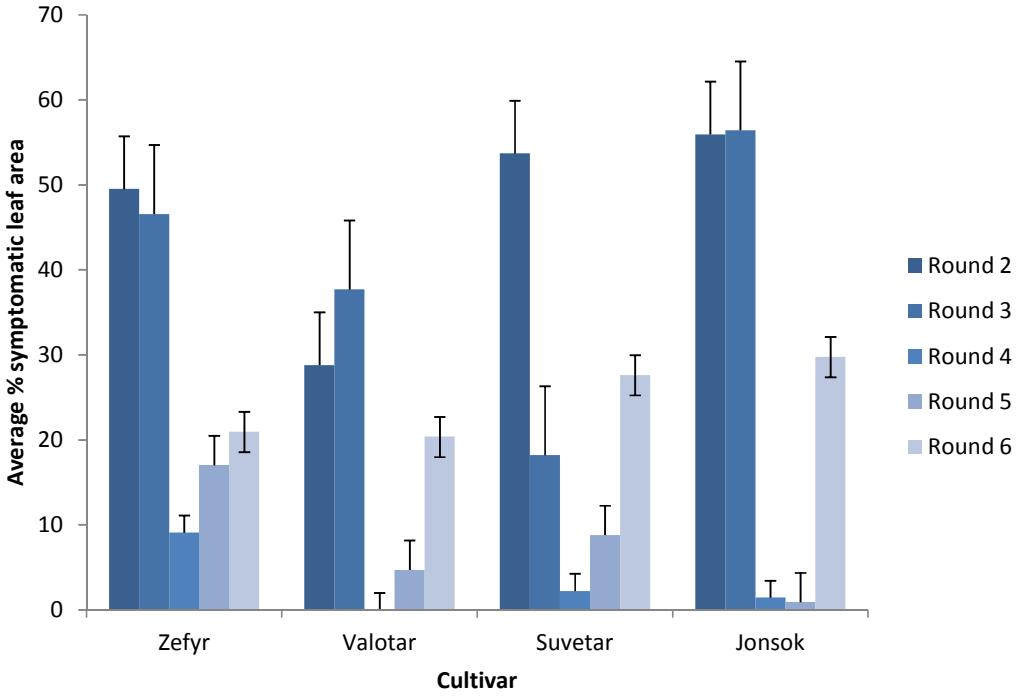

Figure 1. The percentage coverage areas of symptoms of powdery mildew (*P. macularis*) in cultivars of the garden strawberry in the laboratory. The comparison of the means is done separately to each test round. The first test round was rejected, and is not pictured here. The lines above the bars represent the standard error (n = 14 – 21).

On the second test round there was no notable difference between Zefyr, Suvetar and Jonsok, but Valotar had generally less symptoms than the others. On the third round the

differences between cultivars were clearer. Again, there were most symptoms on the leaflets of Jonsok and second most on Zefyr. On this round it was Suvetar that had the least visible symptoms and Valotar the second least.

On the fourth and fifth round there was much less symptoms on the leaflets compared to the second and third rounds. On both rounds Zefyr showed the most symptoms. The rest of the cultivars showed basically very little symptoms on both of the test rounds. On the sixth test round the amounts of symptoms were very similar between Jonsok and Suvetar. Zefyr and Valotar also had very similar amounts of symptoms, but slightly less than Jonsok and Suvetar.

Even though the results varied greatly between test rounds, when the results were examined together an overall trend could be noticed. When the data of all test rounds were put together, on average Jonsok and Zefyr seemed to show more symptoms than Valotar and Suvetar (Figure 2). When an ANOVA single factor data analysis was performed on the results, it was found out that the difference between the cultivars were statistically significant (Table 3). When the test groups were compared to each other, it was found out that the difference between Jonsok was statistically significant with Valotar ($p= 0.022$) and Suvetar ($p= 0.028$).

Even though the average result of Zefyr did not differ statistically from Jonsok ($p= 0.589$) and seemed to be notably higher than the result of Valotar and Suvetar, the statistical significance failed to be proofed ($p= 0.070$ and $p= 0.092$, respectively). Valotar and Suvetar did not differ from one another significantly ($p= 0.839$).

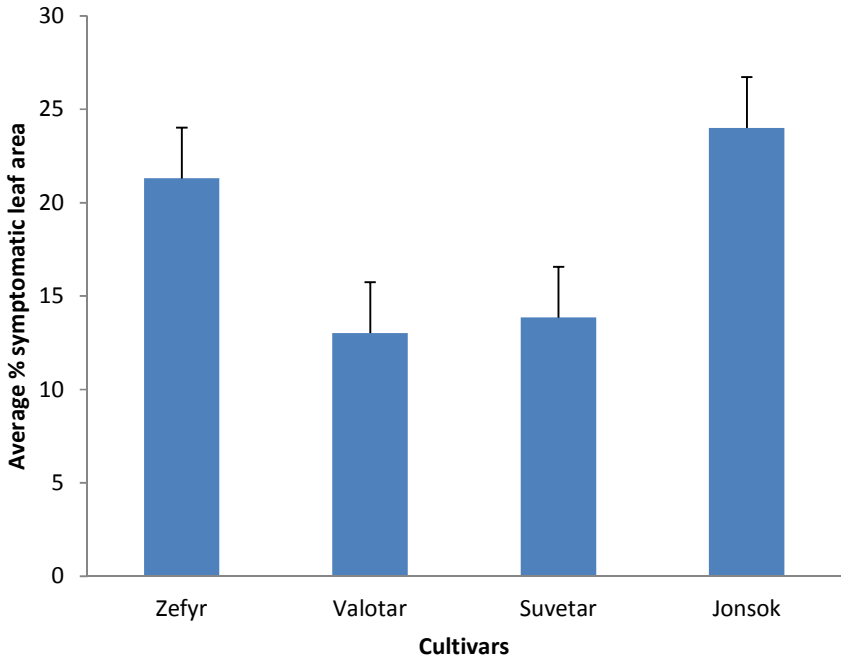

Figure 2. The percentage coverage areas of symptoms of powdery mildew (*P. macularis*) in cultivars of the garden strawberry. The results of five test rounds are added together, and their means are compared. The lines above the bars represent the standard error (n = 70 – 77).

Table 3. The ANOVA single factor data analysis on the amounts of symptoms of powdery mildew (*P. macularis*) on the garden strawberry in the laboratory. The amounts of symptoms are compared between four cultivars.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6671.48	3	2223.83	2.8331	0.03853	2.635
Within Groups	233129	297	784.946			
Total	239800	300				

SS= sum of squares
df= degrees of freedom
MS= mean square

In all of the cultivars and on all test rounds the sample leaflets begun to turn red after a few days of incubation. Differences occurred between the cultivars on both the samples inoculated with powdery mildew and the negative controls (Figure 3).

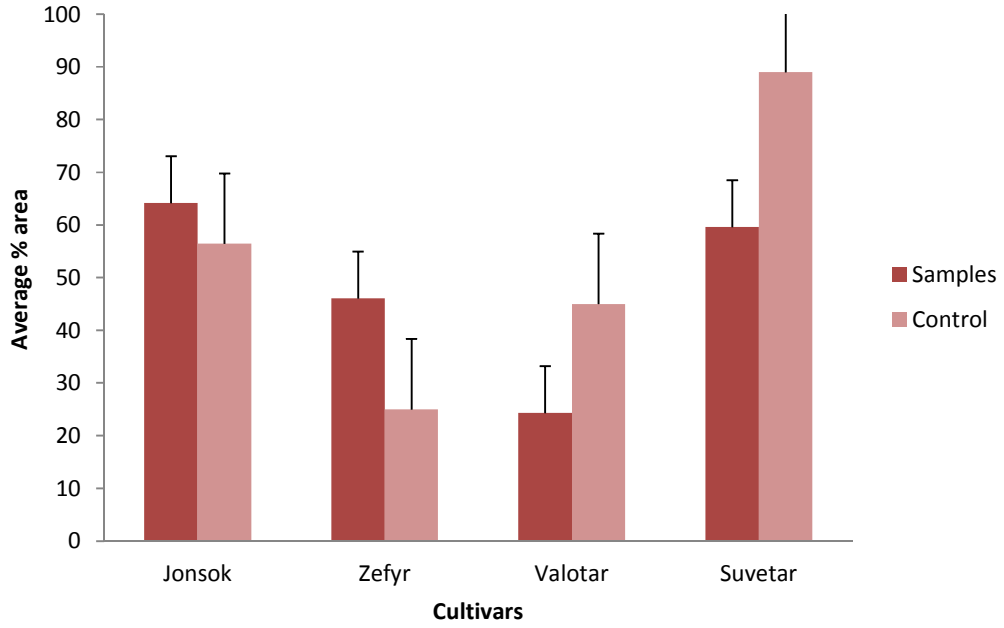

Figure 3. The percentage coverage areas of redness in cultivars of the garden strawberry after two weeks of inoculating the samples with conidia of *P. macularis*, compared to un-inoculated negative controls. The means are compared. The lines above the bars represent the standard error (n = 21).

5.1.2. Greenhouse tests

The difference in the relative resistance to powdery mildew between cultivars in the greenhouse conditions was tested by moving *in vitro*-grown strawberry plants into soil and exposing them to the disease pressure in the greenhouse. The symptoms were then screened on the plants after four, six and eight weeks.

After the plants were moved from roofed mini greenhouses to open tables in the greenhouse, sporulation of powdery mildew could be seen on the leaves only after a couple of weeks. At the time of the first round of measurements after four weeks of exposure to the disease pressure in the greenhouse some of the plants were heavily diseased with white, powdery like sporulation on the leaves. By the time of the second measurements after six weeks some individual plants were completely covered by the fungus.

After eight weeks of being exposed to continuous disease pressure from surrounding plants, some of the smallest plants had died. Most of the plants seemed, however, to have regained their vitality. At this point most of the new leaves did not show visible symptoms even though fully emerged, and the amount of sporulation on some of the older, previously heavily infected leaves had decreased. After approximately 12 weeks from first exposure in the greenhouse the epidemic seemed to have decreased notably. On top of the leaves there were only some blotches of sporulation here and there. Some of the plants had some sporulation still occurring on the abaxial surfaces.

The results of measurements on individual leaves varied greatly during the greenhouse tests on all cultivars. On one plant there could be leaves with 0 to 100% of their surface area covered with sporulation. During the four weeks of observation the amounts of disease changed notably in the plants (Figure 4). There were statistically significant differences in the amounts of disease between the cultivars after four and eight weeks after the exposure to the greenhouse conditions (Tables 4 and 5, respectively).

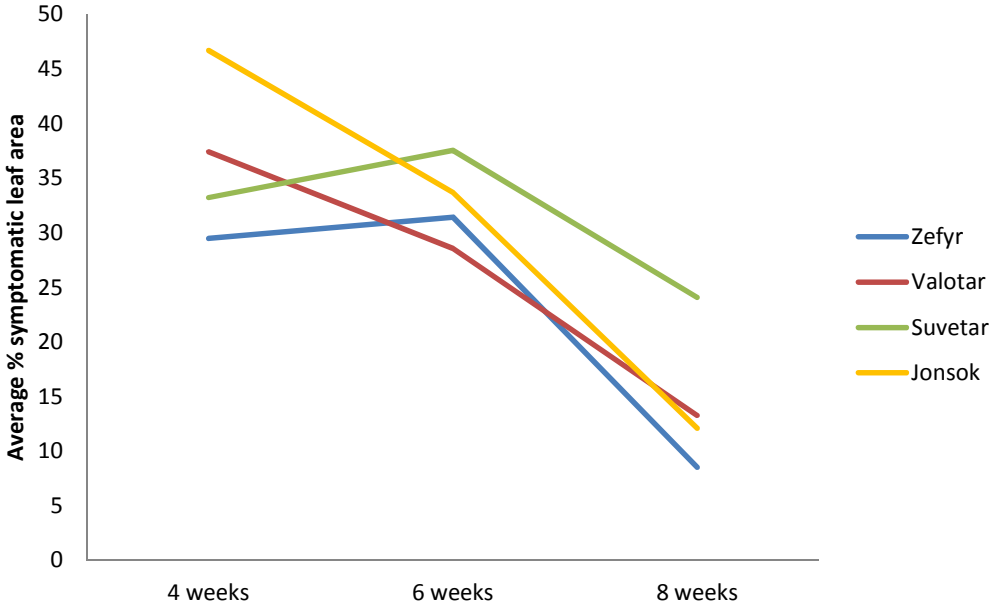

Figure 4. The percentage coverage areas of leaves showing symptoms of powdery mildew (*P. macularis*) in cultivars of the garden strawberry in the greenhouse. The comparison of the means is done separately to the results of four, six and eight weeks.

Table 4. The ANOVA single factor data analysis on the amounts of symptoms of powdery mildew (*P. macularis*) on the garden strawberry in the greenhouse, after four weeks of exposure. The amounts of symptoms are compared between four cultivars.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	32247.3	3	10749.1	9.63608	3.03E-06	2.61694
Within Groups	825473	740	1115.5			
Total	857720	743				

SS= sum of squares
df= degrees of freedom
MS= mean square

Table 5. The ANOVA single factor data analysis on the amounts of symptoms of powdery mildew (*P. macularis*) on the garden strawberry in the greenhouse, after eight weeks of exposure. The amounts of symptoms are compared between four cultivars.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	19196	3	6398.67	15.5369	1.1E-09	2.62314
Within Groups	201388	489	411.837			
Total	220585	492				

SS= sum of squares
df= degrees of freedom
MS= mean square

The average amounts of disease in the four cultivars differed greatly at the time of the disease assessments (Figure 4.) After four weeks of exposure in the greenhouse the average amount of disease on seedlings of Jonsok was significantly bigger than on Suvetar ($p<0.001$), on Valotar ($p= 0.027$) and on Zefyr ($p<0.001$). There were no statistically significant differences between Valotar and Suvetar ($p= 0.28$), Valotar and Zefyr ($p= 0.059$) or Zefyr and Suvetar ($p= 0.247$). After six weeks the amounts of symptoms had generally increased on Suvetar and Zefyr and decreased on Valotar and Jonsok, but no significant differences in the amounts of the disease between the cultivars ($p= 0.848$) could be detected at this time.

At the time of final measurements after eight weeks of exposure the amounts of disease on all four cultivars had decreased. The average amount of the disease on Suvetar was significantly bigger than on Jonsok ($p<0.001$), Valotar ($p=0.0073$) and on Zefyr ($p<0.001$). Jonsok and Valotar had almost the same average amount of disease ($p= 0.65$), and Zefyr had the least with a statistically significant difference to Jonsok ($p= 0.025$) and Valotar ($p= 0.05$).

When the results of the greenhouse tests were compared to the put-together data of the laboratory tests, no consistency could be observed. It was also observed, that visible sporulation could not be seen on the un-emerged leaves, even if the rest of the plant was completely covered with fungal growth (Figure 5).

Figure 5. A heavily diseased seedling of Suvetar after six weeks of exposure to the disease pressure of powdery mildew in the greenhouse. Un-emerged leaf is lacking visible sporulation even though the rest of the plant is completely covered by fungal growth (red arrow).

5.2 Experiments on wild strawberry

5.2.1 Laboratory tests

The laboratory tests on the F_2 crossing populations of *F. vesca* failed to give any results. The pathogen failed to produce any visible symptoms of powdery mildew, such as conidia and conidiophores on all of the samples. Even though many of the leaflets started to decompose very early, and despite regular moistening of the filter papers some of the leaflets died of drought, most of the sample leaflets appeared to be in good condition after the two weeks.

5.2.2 Greenhouse tests

The assessment of the differences in powdery mildew resistance between the plant individuals in the greenhouse was tested on three F_2 crossing populations of the wild strawberry (*F. vesca*). Parental accessions were grown as controls. The germination level of the parental accession H4 was so poor, that only ten plant individuals managed to grow big enough to be assessed. The symptoms were then screened on each plant individually, taking into account the emerging order of the leaves

When the plants were relocated into the short day room, and the plastic was removed, the first symptoms of powdery mildew could be seen after three weeks. The first measurements were done right after the first symptoms had appeared. During the first measurements the differences in the sizes of the plants were quite big. This made it more difficult to compare the measurements between the plant individuals and the crossing populations. Because of this, the assessment of the symptoms was repeated after two weeks.

It was noticed that the general trends implied by the first results were reinforced by the results of the second assessment. At the time of the second assessment the amounts of emerged leaves had become more even, and the data was easier to analyze and compare between populations. For this reason, only the measurements of the second assessment were further analyzed. Leaving out a few individual exceptions, the plants in both H4 x Baron and Fv x H4 -populations had five fully emerged leaves. Almost all the plants in the Fv x Baron -population had four fully emerged leaves. At this point none of the first leaves had yet started to die, so the progress of their symptoms could still be taken into account.

When the percentage symptomatic areas of individual plants were calculated it was noticed that there were clear differences in the levels of contamination between the plant individuals. When histograms were made, it was noticed that in every population the distribution of the plant individuals with different contamination levels would roughly comply with Gaussian distribution. However, differences could be seen in the place of the highest peak of the distribution between the populations (Figure 6). Fv x H4 -population seemed to have more contaminated plant individuals than H4 x Baron and

Fv x Baron -populations. The Fv X Baron -population seemed to have the smallest amounts of contamination in the plants.

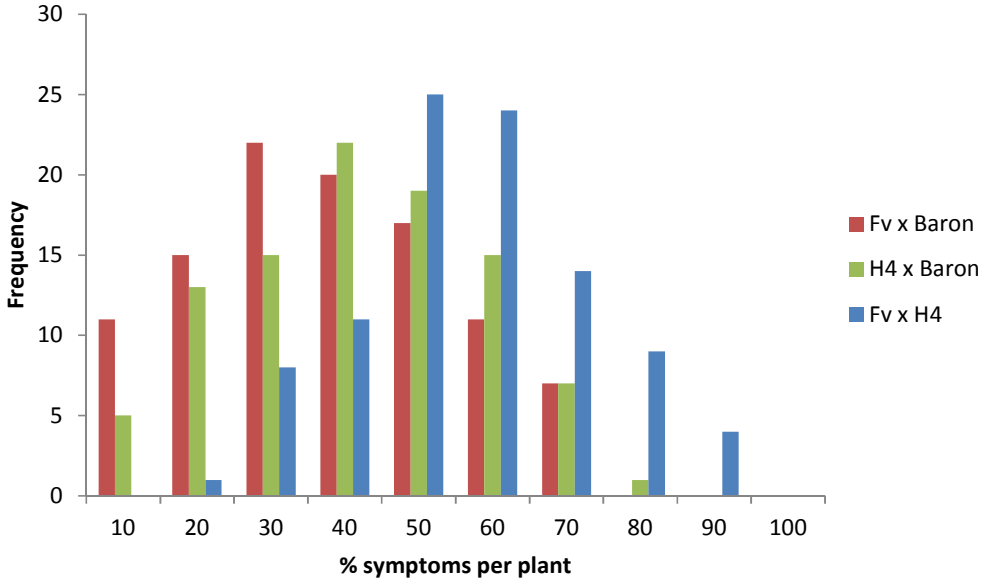

Figure 6. The frequency and the distribution of the individual plants of F_2 crossing populations of the wild strawberry based on the mean percentage amounts of visible symptoms of powdery mildew, after five weeks of exposure in the greenhouse (n = 98 – 105).

Differences between individual plants could also be seen from the control group of parents (Image 7). The distribution of the control plants did not follow the Gaussian distribution that clearly. However, a general trend could be observed where the Baron plants seemed to have less contaminated plants than H4 and Fv. H4 might have been more susceptible than Fv, but this can only be speculated because the amount of test plants was so small.

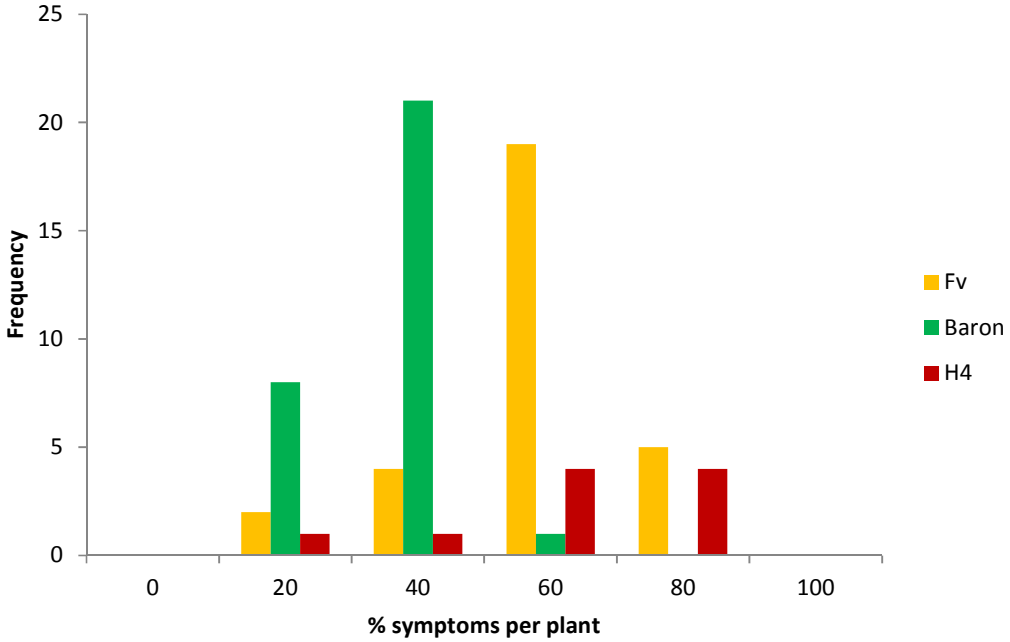

Figure 7. The frequency and the distribution of the individual plants of parental accessions of the wild strawberry based on the mean percentage amounts of visible symptoms of powdery mildew, after five weeks of exposure in the greenhouse (*n* Fv and Baron = 30, *n* H4 = 10).

The first two or three leaves to emerge seemed to end up with bigger surface area covered by powdery mildew in all of the three populations (Figure 8). When the two populations with five fully emerged leaves (H4 x Baron and Fv x Baron) were compared, it was observed that generally the Fv x Baron -population seemed to have more visual symptoms on the leaves that the H4 x Baron -population. In all three populations it was noticed that the newer fully emerged leaves never became as contaminated as the first emerged leaves.

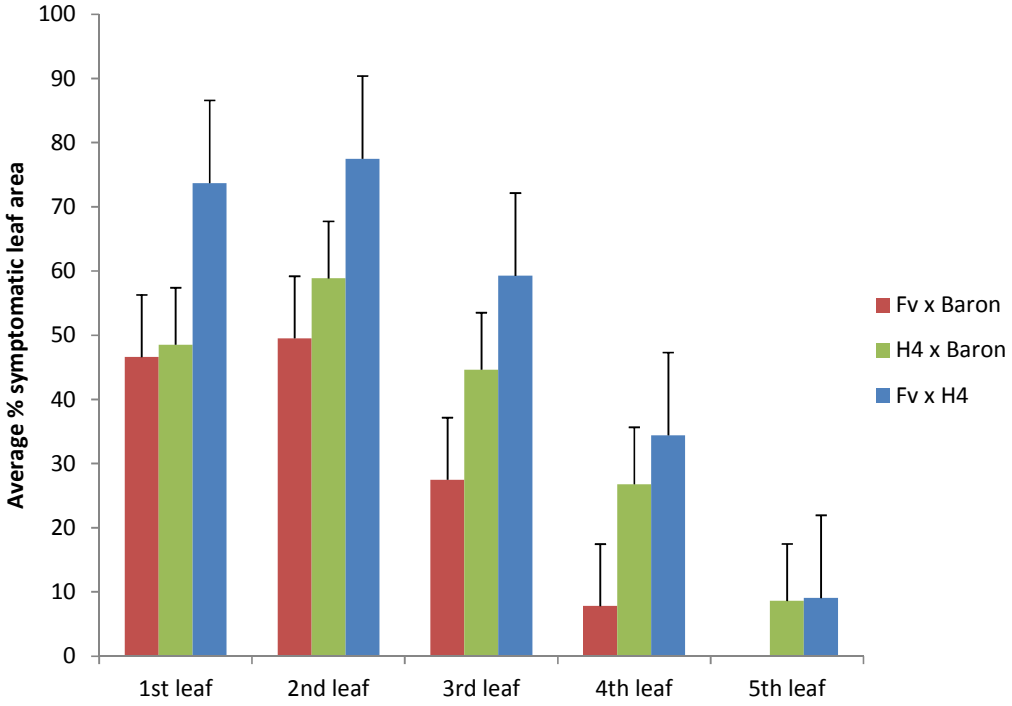

Figure 8. The percentage coverage areas of symptoms of powdery mildew in leaves of the wild strawberry in the greenhouse, after five weeks of exposure. The comparison of the means is done separately to each leaf, in the order of their emerging. The lines above the bars represent the standard error (n = 98 – 105).

5.2.3 The chi-square test

The connection between two genetically controlled traits, the production of runners and the flowering habit, and the ability of the plant individuals to resist the infection of powdery mildew was tested. The production of runners was observed from plants of F_2 crossing populations of Fv x Baron and H4 x Baron, grown in the greenhouse. The habit of flowering was defined from DNA samples extracted from plants of F_2 populations of Fv x Baron and Fv x H4. The ratio of the expressed phenotypes was tested with a chi-square test.

There were no statistically significant differences in the ratios of the expression of runner production in either in the groups of least symptomatic nor the most symptomatic plant individuals (Table 6).

Table 6. The chi-square test for the ratio of the expression of runner production in the groups of plants showing most (n = 30) and least (n = 30) symptoms to powdery mildew, in F_2 crossing populations of Fv x Baron and H4 x Baron of the wild strawberry.

A Most symptomatic		B Least symptomatic	
Observed values	**Expected values**	**Observed values**	**Expected values**
20 producing runners	22.5 producing runners	25 producing runners	22.5 producing runners
10 no runners	7.5 no runners	5 no runners	7.5 no runners

Most symptomatic		Least symptomatic	
χ^2= 1.11111	Chi-square value: 0.46	χ^2= 1.11111	Chi-square value: 0.46
df= 1		*df*= 1	
p= 0.5		*p*= 0.5	

There were also no significant differences in the ratios of expression of the flowering habit in either of the groups (Table 7). The extraction of DNA failed from some of the samples, thus the chi-square test was performed on 25 of the least, and on 19 of the most contaminated plant individuals.

Table 7. The chi-square test for the ratio of the expression of flowering habit in the groups of plants showing most (n = 25) and least (n = 19) symptoms to powdery mildew, in F_2 crossing populations of Fv x Baron and Fv x H4 of the wild strawberry.

A Most symptomatic		B Least symptomatic	
Observed values	**Expected values**	**Observed values**	**Expected values**
16 seasonal flowering	14.25 seasonal flowering	21 seasonal flowering	18.75 seasonal flowering
3 perpetual flowering	4.75 perpetual flowering	4 perpetual flowering	6.25 perpetual flowering

Most symptomatic		Least symptomatic	
χ^2= 1.235746	Chi-square value: 0.46	χ^2= 1.08	Chi-square value: 0.46
df= 1		*df*= 1	
p= 0.5		*p*=0.5	

6 DISCUSSION

6.1 Experiments on garden strawberry

6.1.1 Laboratory tests

The results of the laboratory tests did not give an unambiguous conception of the practicability of the methods used in assessing the relative resistance to powdery mildew in laboratory. There were great differences between the test rounds. The first test round had to be disqualified completely because only one of the total 84 leaflets inoculated showed any measureable symptoms. When compared to each other, the other five test rounds did not seem to be consistent. On each of the test rounds the terms in

amounts of visible symptoms were different between the cultivars, and a general trend was difficult to point out.

When the data of all the test rounds that gave any results was put together there could, however, be seen a trend that indicated the differences in the resistance to powdery mildew between the cultivars. This was congruent with the prior knowledge on the field resistance of the cultivars to powdery mildew. As hypothesized, the more susceptible cultivars Zefyr and Jonsok generally showed more symptoms than the more resistant Valotar and Suvetar.

Several factors might have contributed to the inconsistent results of the laboratory experiments, and the heterogeneity between the test rounds. First of all, even though the inoculations were done with caution and care, it was very difficult to make sure the same amounts of inoculum landed on each of the leaflets. As a result, more conidia most likely ended up on some of the leaflets than the others. In their study on controlled inoculation with *Erysiphae graminis*, Nair and Ellingboe (1962, ref. Nicot and Dik 2002) pointed out that large clumps and high densities of spores may result in lower rates of germination compared to smaller amounts of spores. They also noticed the difficulties in quantifying and unifying the amounts of inoculum when dusting conidia onto the leaves. This is probably one of the reasons why – against all expectations – visible amounts of inoculum brushed onto the leaflets did not always lead to clear symptoms, even on the less resistant cultivars Jonsok and Zefyr.

The endurance of the leaflets during the incubations also proved to be a problem. Not all of the leaflets survived the approximately two-week incubation period. Even though it was intended to use leaves of the same age in the experiments, small variation in the sizes and ages of the leaflets could not be avoided. As a result of this, the smallest leaves dried more easily, and many had to be discarded. Some of the bigger leaflets on the other hand, started to decompose after about a week of incubating. Due to its biotrophic nature, the powdery mildew did not grow on these necrotic areas, and this could have distorted the results. This problem was also encountered by Peries (1962b), who used similar methods of inoculation and incubation in his studies on the strawberry powdery mildew.

Concerning also the endurance of the leaves, keeping the conditions stable inside the Petri-dishes proved to be difficult. Because the Parafilm was not used to insulate the dishes, the filter paper needed to be watered every couple of days to keep the conditions moist enough for both the detached leaflets and the fungus. Estimating the optimal time span for the watering was difficult even after several test rounds. Even though the amount of water used on each watering was kept as constant as possible, some dishes always seemed to be too wet and some too dry.

Jhooty and McKeen (1965) found out that on host surfaces the germination of the spores of powdery mildews can occur even under very dry conditions. This suggests that the dry conditions in some of the Petri-dishes might not have been a problem to the fungus, had the leaflets stayed alive. Detached from the plant the smallest leaflets did not endure the dry conditions for even a day. Too wet conditions on some of the dishes might, on the other, hand have been harmful to the fungus. It has been shown that relatively short periods (>5 hours) of immersion had a lethal effect on the conidia of *P. macularis* (Peries 1962a). Even though it was made sure that during the watering of the filter papers water droplets did not end up on leaf surfaces, too wet conditions cannot be ruled out as one factor having contributed to the inconsistency of the results.

It was described by Corke and Jordan (1978), that red blotches appear on the strawberry leaves as the disease progresses. Even though red areas were observed, and there seemed to be differences between the cultivars in the redness of the leaflets after the incubation, differences also occurred between the non-inoculated negative samples. No common factor between the redness and the presence of powdery mildew could be detected.

The results of the laboratory experiments suggest that the used methods need some adjustment in order to give clearer results. The biggest shortcoming of the method was that it was not managed to keep the conditions for the inoculum and the incubation stable and identical. It seems that the critical factors were the amounts of inoculation landing on each leaflet, and the moisture conditions inside the Petri-dish.

Nair and Ellingboe (1962 ref. Nicot and Dik 2002) solved the problem of uneven amounts of inoculum by first shaking the conidia from diseased leaves to a glass slide and then removing the clumps of conidiophores by blowing. For mass inoculations

Peries (1962a) placed the plant material to be inoculated at the bottom of a cardboard cylinder, and brushed the conidia from infected material at the top of the cylinder. For more precise work he used an eyelash attached to a glass needle to inoculate leaves with single spores. Single spore inoculations need a lot of work, and are thus not useful in large amounts of samples.

Making the humidity conditions stable inside the Petri-dishes is an aspect that may need more methodological studies. It can be that if the experiment was performed on bigger leaves, the plant material would better have survived the two week incubation period. With *in vitro* plants this might be a problem, because it would take more time and procedures, such as transplanting to bigger containers, to grow the seedlings big enough to produce more durable leaves.

Even though the results of the laboratory experiments were not as unambiguous as hypothesized, the general trend of the data suggests that the method might give a useful hint to the relative resistance to powdery mildew of new strawberry cultivars. It is, however clear, that without further studies and adjustments the method is not yet ready to be used as a quick-test to determine powdery mildew resistance.

6.1.2. Greenhouse tests

The results of the greenhouse experiment did not give a clear picture of the differences in the resistance to powdery mildew between the cultivars. Even though statistically significant differences could be seen between the cultivars at the times of disease assessments, the terms between the cultivars in the amount of visible symptoms were different at the time of all the three disease assessments. Against the hypothesis, the susceptible cultivar Zefyr had generally least visible symptoms during the observations, and thus seemed to be most able to resist the disease. The biggest drop in the amounts of disease during the experiments could be seen on the other susceptible cultivar Jonsok. The only general trend that could be clearly observed from the development of the epidemic during the experiment was that after eight weeks the amounts of visible symptoms had decreased from the time of the first assessment on all of the four

cultivars. Unlike on Zefyr and Suvetar, on Jonsok and Valotar the amount of disease was lower already at the time of the second assessment.

It was hypothesized, that the cultivars Jonsok and Zefyr would be clearly more susceptible to the infection of powdery mildew in the greenhouse than the more resistant cultivars Valotar and Suvetar, because their susceptibility has been noticed in practical field cultivation. This could not be clearly pointed out from the results of the greenhouse experiment. The results have a consistency to previous studies. In Peries' (1962b) greenhouse experiments it was noticed, that in greenhouse conditions field resistance can be overridden, because the humidity is highly favorable to the pathogen. Maas (1984) also noted in his book the un-success of screening seedlings for resistance under glasshouse conditions. Nelson et al. (1996) however suggested in their comparative study, that greenhouse screening could provide a tool to identify field resistant strawberry genotypes.

The controversies between this study and the studies of Peries (1962b) and Nelson et al. (1996) may be explained by the differences in the conditions in the greenhouse. Agricultural practices can go a long way in the control of powdery mildews in the greenhouse environment. The high humidity favorable to the spread of the disease can be controlled with adequate ventilation, and removing the diseased plants will delete the source of the infection. This experiment was done in the greenhouse in Viikki where powdery mildew has caused a lot of trouble. It might be that the lack of agricultural practices to make the conditions less favorable to the disease caused such great disease pressure that even the more resistant cultivars were heavily infected.

Because the greenhouse experiment gave no reference to the prior knowledge on the ability of the four cultivars to resist the infection of powdery mildew in field conditions, there was no consistency to the laboratory results either. The only notion in common to the laboratory experiments was the amounts of disease varied from 0 to 100% on all cultivars during the tests. This suggests that the age of the leaf should be taken into consideration when assessing the disease, because it might be an important factor in expression of the symptoms. This has previously been noticed by Peries (1962b) and Okayama et al. (1995), who pointed out that youngest leaves were most susceptible to the disease. In contrast to the general perception that the powdery mildew is most likely found on the abaxial surface of the leaves (Maas 1984), most of the sporulation

observed during the experiment was on the upper surfaces of the leaves. Gadoury's (et al. 2007b) study supports this by pointing out that there is no difference in susceptibility of the upper versus lower leaf surfaces.

It was also noticed, that even on the plants completely covered with white powdery growth of the fungus, the un-emerged leaves were bright green and not showing symptoms. Even though this seemed like a prominent point, it was probably happening only because the leaves had emerged faster than the fungus was able to start the production of conidia. It was pointed out by Corke and Jordan (1978) that in optimal conditions on strawberry the production of conidia begins four days after completing the penetration of host tissues.

Even though the hypothesis could not be fulfilled, this experiment still provided more information on the process of the powdery mildew epidemic in humid greenhouse conditions. From the point of view of evaluating the method it was noticed, that in order to get clearer results, adjustments need to be done on the conditions in the growth room. Perhaps relative resistance of strawberry cultivars could still be evaluated in the greenhouse, if the conditions were more controlled. In further studies this aspect could be tested by for example in small, ventilated rooms in a way that the disease would origin from a controlled dose of inoculum, and no continuous disease pressure would be present from outside the test plants.

6.2 Experiments on wild strawberry

6.2.1 Laboratory tests

No self-evident reason for the complete failure of the laboratory tests on the wild strawberry can be pointed out. The experiment was carried out similarly to the tests done on garden strawberry. Why there was absolutely no signs of powdery mildew on any of the 50 Petri-dishes could have had something to do with the quality of the inoculum. Corner (1935) noticed in his studies on the resistance to powdery mildews,

that the average life span of powdery mildew conidia is from 2 – 3 days to a week, and sample taken from an old patch of mycelium will always give low percentage germination. The effect of the age of the conidial germination rate was also noticed by Peries (1962a).

The inoculum for the laboratory test on wild strawberry was collected in the middle of the summer when the powdery mildew epidemic seemed to be at a low point in the greenhouse. It is probable that the fungus was in a more or less latent stage in the plants, and the conidia brushed to the leaflets on the Petri-dishes were not alive anymore. From this it can be concluded, that if inoculum is received from an infected plant, it must be made sure the sporulation on the plant is fresh.

6.2.2 Greenhouse tests

The experiments in the greenhouse indicate that there are differences between the phenotypes of individual plants in all of the three F_2 crossing populations. Variation in degrees of infection was also noticed in *F. vesca* -populations by Harland and King (1957). The histogram (Figure 7) also shows that the means of the three populations are slightly different. The distribution of the plants in the parental accessions implicate that Baron might be most resistant to powdery mildew, and H4 the least.

When the distribution of the plants in the parental accessions are compared to the crossing populations, it can be seen, that the presence of the Baron -genes may contribute to the powdery mildew resistance. This means that Baron seems to be able to transmit its resistance to its progeny. Daubeny (1969) crossed relatively resistant strawberry cultivars with relatively susceptible cultivars, and noticed that the resistance of parents can be transmitted to progenies. Similar notification was made by MacLachlan (1978). These suggestions are supported by Hsu et al. (1969) who proved that resistance to powdery mildew can be explained by parental contribution in most cases. Daubeny (1961) however also noticed that in some cases the resistance genes of the parents are not transmitted to the progenies.

In all of the crossing populations the nature of the frequency distribution could be illustrated as a curve that roughly complies with Gaussian distribution. This is in controversy with Daubeny's (1961) results, whose crossing populations of cultivars of the garden strawberry fell into two classes indicating segregation into resistant and susceptible lines when a relatively resistant cultivar was crossed with a relatively susceptible cultivar.

The differences in the resistance to powdery mildew between the crossing populations were also demonstrated when considering the symptoms on individual leaves. The differences in the amounts of symptoms between the leaves of different ages can probably be explained by the fact that the fifth and fourth leaves simply did not have enough time to develop as severe symptoms by the time of the second observation as the older leaves. The first three leaves to emerge seemed to develop severe symptoms faster than the later emerged, but this was probably due to their smaller size.

In the prospect of evaluating the method used in assessing the symptoms, the results indicate that the method is more or less working. Since the distribution of the data roughly follows the normal distribution, as can be expected from a study of a quantitative trait in a large population, it can be stated that a quick and subjective assessment of percentage disease symptoms on the leaves of strawberry can give relatively accurate results.

It can be stated that the estimation of the percentage of symptomatic leaf area can provide accurate information, but often needs training of the evaluators (Nicot et al. 2002). Since in this study the disease assessment was done without any training for the method, it might be that accuracy of the results was due to the fact that the assessment was done on each leaf individually and the data then summed to obtain the percentage symptoms on the whole plant, rather than assessing the whole plant on single glance. Other significant factor was probably that the assessment was done by a single person, and thus even though the data is very subjective, it is still comparative. Thus, the method used here could perhaps be further adapted into the process of breeding for field-resistant cultivars. This is supported by the study by Nelson et al. (1995) who showed that even though different genes may confer resistance with different levels of disease pressure – in practice meaning in different agricultural systems – field evaluations correspond well with the data obtained from the greenhouse.

6.2.3 The chi-square test

The chi-square test did not show any significant differences in the ratios of expressing the production of runners. Even though no statistical significance could be proved, some differences could, however, be seen between the observed and expected values in both the most and least symptomatic groups. Generally, the group of most symptomatic plant individuals had less, and the least symptomatic group had more runner producing plants than was expected based on the ratio of 3:1. It can be speculated, that there might yet be a weak connection between the ability to resist powdery mildew and runner production. The amount of the individuals in the test groups might have been too small to demonstrate this statistically. Perhaps the linkage between runner production and powdery mildew resistance could be investigated further in the future with larger test groups.

No hint of a linkage between the flowering habit and powdery mildew resistance could be demonstrated by the chi-square test. Simpson (1987) made the same observation when studying the inheritance of powdery mildew resistance in ever-bearing and day-neutral seedlings of garden strawberry. He found no evidence that crosses involving ever-bearing traits would behave differently from those not involving them in their ability to resist powdery mildew.

7 CONCLUSIONS

The aims of this study were to investigate if there would be a quick and simple way of testing for field resistance of strawberry to powdery mildew, and to compare the compatibility of the results of disease assessment for relative resistance done in the laboratory and in the greenhouse. In addition, the inheritance of powdery mildew resistance was observed on seedlings of F_2 crossing populations of three varieties of wild strawberry, to find out if the differences in the relative resistance could be linked to a phenotypic character.

The results of this study were not unambiguous, but yet useful practical information was obtained on the study methods, their comparability, and the behavior of *Podosphaera macularis* in different conditions. The main concern in the success of tests done in the laboratory is the quality of the inoculum, age of the plant material and uniform conditions throughout the experiments. Even though in this study the laboratory tests were not a great success, it can be suggested that with conformed and coherent methodology it is possible to get at least directional idea of the field resistance of different cultivars.

It was noticed, that even though the laboratory tests were able to give a hint on the field-resistance of the cultivars of the garden strawberry, it might be difficult to get similar results in the greenhouse. The solving of this problem could be a topic for further investigation. The results of this study, with plenty of references in the literature show that the powdery mildew can behave unexpectedly in many kinds of environments and that with some adjustments to the study situation this problem could be overcome.

Even though no statistically significant information was found on the connection between the ability of the wild strawberry to resist the powdery mildew and runner production or the flowering habit, a hint of a possible linkage was found. In addition, the near Gaussian distribution of frequency of all three crossing populations showed that the subjective assessment of powdery mildew symptoms can be useful in surveying the attributes of plant populations in the greenhouse.

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APPENDIX 1

Rooting medium:

4.32 g/l Murashige and Skoog medium (MS salt)
20 g/l saccharose
6.5 g/l plant agar
pH 5.5 – 5.7

1. MS salt and saccharose are measured and dissolved into MQ water
2. pH is regulated to 5.5 – 5.7.
3. Agar is added and mixture is heated to boiling point.
4. Mixture is poured into lidded jars (60 ml/ jar)
5. Jars are autoclaved.

Germination medium:

2.16 g/l Murashige and Skoog medium (MS salt)
5 g/l saccharose
6.5 g/l plant agar
pH 5.5 – 5.7

1. MS salt and saccharose are measured and dissolved into MQ water
2. pH is regulated to 5.5 – 5.7
3. Agar is added and mixture is heated to boiling point.
4. Mixture is poured into glass container and autoclaved.
5. Autoclaved mixture is poured on Petri-dishes
6. Let cool down and solidify.

If not used immediately, stored inside a plastic bag.

APPENDIX 2

Surface strerilization of seeds:

70% A-ETOH
Tween
2% Na-hypochlorite
Autoclaved water

1. Put seeds on 1,5 ml Eppedorf tubes
2. Add A-ETOH, shake the tubes for 1 minute and pipette out the liquid
3. Add one drop of Tween to Na-hypochlorite, add to tubes and shake, wait 10 – 15 minutes and pipette out the liquid
4. Add autoclaved water, shake vigorously, pipette out the water. Repeat five times.

APPENDIX 3

Isolation of plant DNA from fresh tissue:

CTAB-buffer:

5.0 g 2% CTAB
70 ml 1.4M NaCl
10 ml 20mM EDTA
25 ml 100mM Tris, pH 8
2.5 g 1% PVP-K30 (Fluka)

1. Turn on the waterbath (60^{o}C) and place the CTAB-buffer to pre-warm. Add β-mercaptoethanol to CTAB-buffer to a final concentration of 0.2%.
2. Grind a small leaf of fresh plant material to a powder in liquid nitrogen in a chilled mortar and pestle. Move the powder directly into a 1.5 ml Eppendorf tube. Do not allow thawing before next step.
3. Add 0.5 ml of warm (60^{o}C) CTAB-buffer. Incubate samples at 60^{o}C for 30 minutes and swirl occasionally.
4. Add 0.5 ml of chloroform-isoamylalcholol (24+1), mix gently but thoroughly. Centrifuge for 10 minutes at maximum speed at room temperature. Remove the aqueous phase from the top into a clean tube and repeat the extraction with chloroform-isoamylalchohol until the aqueous phase is clear.
5. Precipitate DNA by adding 2/3 volumes of cold isopropanol. If you do not get a clear precipitate at this stage, you may leave the sample at room temperature for several hours to overnight.
6. Centrifuge for 10 minutes at maximum speed at 4^{o}C. Remove the supernatant carefully.
7. Wash the pellet with 1 ml of 70% ethanol. Remove the ethanol and air-dry the pellet until it becomes transparent.
8. Re-suspend the pellet in 50 µl TE.

9. OPTIONAL: Add 10 µl RNaseA (1 mg/ml stock). Incubate at 30^{o}C for 30 minutes.
10. Measure DNA concentration in a spectrophotometer, or run an aliquot of the sample on gel.

APPENDIX 4

PCR reaction:

The master mix:

Master mix	x 1	x 65
MQ water	12.0 µl	0.78 ml
Buffer	1.5 µl	97.5 µl
Mg^{2+}	1.2 µl	78.0 µl
dNTPs	0.3 µl	19.5 µl
TFL1_lab	0.3 µl	19.5 µl
PRR7	0.3 µl	19.5 µl
CFVCT010	0.3 µl	19.5 µl
Maxima HS	0.04 µl	2.6 µl
	12.5 µl	
+ DNA	2.5 µl	

The program:

PCR-reaction			
95°C	4 min		
95°C	45 sec		
60°C	45 sec	x 9	- 0.5°C/cycle
72°C	30 sec		
95°C	45 sec		
55°C	45 sec	x 25	- 0.5°C/cycle
72°C	30 sec		
72°C	10 min		
4°C	Incubation		